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Identification of serine/threonine kinase and nucleotide-binding site–leucine-rich repeat (NBS-LRR) genes in the fire blight resistance quantitative trait locus of apple cultivar 'Evereste'‡

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SUMMARY

Fire blight is the most destructive bacterial disease affecting apple (*Malus* × *domestica*) worldwide. So far, no resistance gene against fire blight has been characterized in apple, despite several resistance regions having been identified. A highly efficacious resistance quantitative trait locus (QTL) was localized on linkage group 12 (LG12) of the ornamental cultivar 'Evereste'. A marker previously reported to be closely linked to this resistance was used to perform a chromosome landing. A bacterial artificial chromosome (BAC) clone of 189 kb carrying the fire blight resistance QTL was isolated and sequenced. New microsatellite markers were developed, and the genomic region containing the resistance locus was limited to 78 kb. A cluster of eight genes with homologies to already known resistance gene structures to bacterial diseases was identified and the corresponding gene transcription was verified. From this cluster, two genes were recognized *in silico* as the two most probable fire blight resistance genes showing homology with the *Pto/Prf* complex in tomato.

INTRODUCTION

Erwinia amylovora (Burr.) is the causal agent of fire blight disease, the most destructive bacterial disease affecting apple (*Malus* × *domestica*) and pear (*Pyrus communis*) orchards worldwide. Eradication of infected plants, chemical control with antibiotics or heavy metals, and use of biocontrol agents are the control measures used to contain the propagation of the disease (Johnson and Stockwell, 1998; McManus *et al*., 2002; Paulin, 1996). However, these methods are never completely successful as a result of the difficulty in forecasting the disease because of its epidemiological characteristics. Moreover, the use of antibiotics can lead to the rapid selection of antibiotic-resistant *E. amylovora* strains (Jones and Schnabel, 2000). For all of these reasons, genetic resistance is an important element in the management of the disease, which has received limited attention to date. Quantitative trait loci (QTLs) for fire blight resistance, providing different levels of resistance, have been identified in apple (Calenge *et al*., 2005; Durel *et al*., 2009; Khan *et al*., 2006; Peil *et al*., 2007). The QTL identified by Calenge *et al*. (2005), and confirmed by Khan *et al*. (2006), on linkage group 7 (LG7) of cultivar 'Fiesta' explained about 40% of the phenotypic variation of the length of necrosis after inoculation, whereas the QTL identified by Peil *et al*. (2007) on LG3 of the rootstock genotype *Malus* × *robusta* 5 explained about 80% of the phenotypic variation. However, this resistance has already been overcome by strains of *E. amylovora*, indicating a possible gene-for-gene interaction (Norelli and Aldwinckle, 1986; Paulin and Lespinasse, 1990). Durel *et al*. (2009) identified a resistance QTL on LG12 of the ornamental cultivar 'Evereste', explaining up to 70% of the phenotypic variation observed 14 days post-inoculation, but the precise position of the QTL could not be determined because it was distal to the most external simple sequence repeat (SSR) marker used for mapping. The exact position of the QTL peak has been determined recently by G. Parravicini *et al*. (unpublished work) using a combination of bulked segregant analysis and

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‡Nucleotide and amino acid sequences of all the genes found on the restricted resistance region have been submitted to the EMBL database as Accession No. HQ445899.

amplified fragment length polymorphism techniques. Two new markers were developed and the QTL was localized between Hi23d11y_E and M35TA_256s_E markers. The marker most close to the QTL peak was M45TA_403c_E.

Plant genes conferring resistance to bacterial disease are found to act as complexes and to belong to three of the seven classes characterized by Kruijt *et al*. (2005): class 1, comprising serine/threonine (Ser/Thr) kinase genes; class 2, comprising genes coding for a region of leucine-rich repeats (LRRs), a putative nucleotide-binding site (NBS) and a coiled-coil (CC) domain; and class 5, consisting of receptor-like kinase genes encoding an extracellular LRR region, a transmembrane domain (TMD) and a cytoplasmic Ser/Thr kinase region.

The resistance to bacterial speck of tomato caused by *Pseudomonas syringae* pv. *tomato* is one of the most intensively studied bacterium–plant interactions. The Ser/Thr protein kinase Pto monitors the interaction between the *AvrPto* or *AvrPtoB* effector of *P. syringae* and the NBS-LRR protein Prf. In the case of an interaction of Pto with AvrPto or AvrPtoB, Pto autophosphorylates itself, becomes active and activates Prf and another Ser/Thr kinase, Pti1. This induces a cascade resulting in a hypersensitive response (Gutierrez *et al*., 2010; Tang *et al*., 1996; Wu *et al*., 2004; Zhou *et al*., 1995, 1997). In *Arabidopsis*, the mode of action of the resistance gene complex RPM1–RIN4–RPS2 against *P. syringae* is also well documented. RPM1 is an NBS-LRR protein that confers resistance to bacteria expressing either of the effector proteins AvrRpm1 or AvrB (Grant *et al*., 1995). These effectors are able to induce the phosphorylation of the *Arabidopsis* protein, RIN4, which, in turn, activates RPM1, triggering resistance (Mackey *et al*., 2002). RPS2 is a further NBS-LRR protein from *Arabidopsis* that confers resistance to *P. syringae* expressing the type III effector AvrRpt2 (Bent *et al*., 1994). The target of AvrRpt2 is also RIN4 and causes its disappearance, which, in turn, activates RPS2 and triggers resistance (Mackey *et al*., 2003). In rice, a receptor-like kinase protein, Xa21, which possesses an extracellular LRR, a TMD and a cytoplasmic Ser/Thr kinase region, confers resistance to the pathogen *Xanthomonas oryzae* pv. *oryzae* by sensing the *avr* gene product of the pathogen (Song *et al*., 1995; Wang *et al*., 1998).

In classical apple breeding, with the goal of introgressing missing traits, such as fire blight resistance, into a commercially competitive cultivar, new cultivars with new characteristics have been produced, but this approach is time consuming because of the long juvenile phase and the several generations needed to obtain the appropriate combination of characteristics. Genetic modification of commercial cultivars by the addition of a particular trait is an attractive alternative to classical breeding in highly heterozygous crops such as apple. Cisgenesis, the technology employed to genetically modify plants by leaving in the final product only genes (in the sense orientation) controlled by their native promoter and terminator, from a crossable, sexually compatible, plant (Schouten *et al*., 2006), seems to enjoy greater public acceptance than transgenesis (Lusk and Sullivan, 2002). To date, the only resistance gene cloned in apple is *HcrVf2*, a gene conferring apple scab resistance (Belfanti *et al*., 2004; Gessler *et al*., 2009). As a result of the important damage generated by fire blight, the identification of *Malus*-specific fire blight resistance genes to add to the genome of a popular and commercially successful apple cultivar is therefore highly desirable. The work presented here paves the way to the cloning of apple fire blight resistance gene(s) of apple cultivar 'Evereste' carrying a QTL with major effect. The positional cloning strategy using a bacterial artificial chromosome (BAC) has already been employed successfully to clone single QTLs in various plants (Paran and Zamir, 2003; Salvi and Tuberosa, 2005), and to identify candidate genes inducing resistance to apple scab in the *Vr2* (Galli *et al*., 2010) and *Vf* (Vinatzer *et al*., 1998; Xu *et al*., 2001) resistance regions, which led to the identification of the functional resistance gene *HcrVf2* (Vinatzer *et al*., 2001; Xu and Korban, 2002) and the creation of scab-resistant transgenic plants from a scab-susceptible cultivar (Belfanti *et al*., 2004; Szankowski *et al*., 2009).

In this article, we report the chromosome landing within the region of the fire blight resistance QTL on LG12 of apple cv. 'Evereste' (called Fb_E by Durel *et al*., 2009). The whole region has been sequenced and new markers have been developed and mapped. This allowed the reduction of the sequence in which candidate resistance genes were searched for. Open reading frames belonging to two of the three classes of genes conferring resistance to bacterial diseases have been identified. A comparative analysis of allelic polymorphism and transcription studies of the candidates have also been performed.

RESULTS

Identification of recombinants within the fire blight resistance region

One hundred and three recombinant individuals between markers M35TA_256s_E and Hi23d11y_E, bracketing the QTL, were identified in the whole mapping population, which consisted of a total of 2703 individuals (i.e. 3.8% of recombination). The recombination rate was similar in MxE and ExM populations (data not shown).

Chromosome landing

The purchased apple cv. 'Evereste' BAC library represents 6.9 times the apple haploid-genome equivalent, with a probability of finding any apple gene of greater than 99%, taking into account that the apple genome size ranges between 743 and 769 kb

Table 1 Markers developed from the sequence of the bacterial artificial chromosome (BAC) end.

Name	Primer forward	Primer reverse	Amplicon size (bp)
38-RP	5′-CGGTGGCTAATTGTTGTGTG-3′	5′-ATGCCATTTTGGTTGGAGAC-3′	422
44-RP	5′-GTGTCCACCATCCGTCAAG-3′	5′-TCGAGCAACAACGGAATTAAG-3′	435
89-T7	5′-AGCGTGCTGATAACGTGTTG-3′	5′-GAACAGTAACCATCGGTTTGG-3′	306

(Arumanagathan and Earle, 1991; Clarke and Carbon, 1976). Six clones hybridizing with the M45TA probe derived from the cleaved amplified polymorphic sequence (CAPS) marker M45TA_403c_E (the closest to the QTL peak) were identified during the screening of the BAC library: 3P24, 24N18, 38M12, 44A20, 62G1 and 65M9. The restriction digestion of the CAPS marker M45TA_403c_E revealed that the BAC clones 3P24, 24N18, 38M12 and 44A20 were derived from the resistance region, whereas 62G1 and 65M9 were derived from the homologous region on the susceptible chromosome. An additional screening was performed with a probe derived from the RP BAC insert-end of the 44A20 BAC clone (named 44-RP probe) to identify the complete corresponding susceptible region. Four clones hybridized with slightly different degrees of intensity, 6J2, 48N20, 69G1 and 89D22, with the first two derived from the susceptible chromosome and the last two derived from the resistant chromosome (according to hybridization with the 44-RP probe of the *Hin*dIII fingerprints).

Two new markers developed from the RP end of the BAC clones 38M12 and 44A20 and one new marker from the T7 end of the clone 89D22 were mapped on LG12 of cv. 'Evereste' (Table 1 and Fig. 1). When focusing on the individuals recombining within the BAC clone 44A20, the phenotypic data indicated that the resistance region was restricted to the interval between the 44-RP and M45TA_403c_E markers fully spanned by this BAC clone (Fig. 1). Only one recombinant (ME2-306) exhibited a somewhat ambiguous phenotype (i.e. intermediate between resistant and susceptible), giving a double recombinant when considered as susceptible.

BAC sequencing and fine mapping

A contig of 2900 sequences, with an average length of 538 bp, was generated to fully sequence the BAC clone 44A20. The total length of the BAC sequence was 188 650 bp, with no ambiguities and with a minimum coverage of two sequences (5.4 times coverage on average).

The alignment of the 44-RP and M45TA_403c_E marker sequences on the 44A20 BAC sequence allowed the restriction of the resistance region to 165 kb. To further narrow the size of the resistance region, 15 primer pairs flanking microsatellite repeats were designed. Nine microsatellite markers (Table 2) were polymorphic in the MM106 × 'Evereste' cross and could be mapped on LG12 of 'Evereste'. Six (ChFbE02, ChFbE03, ChFbE04, ChFbE05, ChFbE06 and ChFbE07) co-segregated with the fire blight resistance locus Fb_E, whereas the other three, ChFbE01, ChFbE08 and ChFbE09, segregated at genetic distance of 0.11, 0.07 and 0.19 cM, respectively, from Fb_E (Fig. 1).

The fire blight resistance region was therefore restricted to the 78-kb interval between the ChFbE01 and ChFbE08 markers, having three recombinant individuals between marker ChFbE01 and the fire blight resistance locus (Fb_E) and two recombinant individuals between Fb_E and ChFbE08 markers (Fig. 1 and Table 3). According to all other recombinant individuals, individual ME2-306 could be considered as resistant rather than susceptible.

The alignment of the 48-T7 marker sequence, derived from the susceptible BAC clone 48N20, on the 44A20 BAC sequence indicated that the 48N20 BAC clone was sufficient to span the homologous susceptible region of Fb_E (Fig. 1).

Identification of candidate fire blight resistance genes and analysis of susceptible alleles

The analysis of the 78-kb resistance region using FGENESH software with the tomato genome as a reference (Salamov and Solovyev, 2000) identified 23 putative genes (Fig. 2). Eight belonged to classes 1 or 2 of the three classes of bacterial resistance gene reported previously: protein kinases (seven) and CC-NBS-LRRs (one). The genes predicted to encode protein kinases were named M*alus* d*omestica* cv. Evereste E*rwinia* a*mylovora* kinase genes (*MdE-EaK*), and the CC-NBS-LRR gene was named M*alus* d*omestica* cv. Evereste E*rwinia* a*mylovora* NBS-LRR gene (*MdE-EaN*).

The amino acid lengths of all the putative protein kinases identified ranged from 381 to 584 amino acids. They showed an identity at the amino acid level between each other (Table 4), ranging from 51% (between MdE-EaK3 and MdE-EaK7) to 84% (between MdE-EaK5 and MdE-EaK7). The proteins predicted to be protein kinases were investigated using expasy prosite for the presence of two signatures characterizing the gene family: the Ser/Thr kinase signature and the ATP-binding signature (Hulo *et al*., 2008). Of all the putative protein kinases found, two, MdE-EaK2 and MdE-EaK7, possessed the Ser/Thr kinase signature, but only one, MdE-EaK7, possessed both Ser/Thr kinase and the ATP-binding signature (Fig. 3 and Table 5), making this gene the best candidate for the fire blight resistance gene among the seven putative protein kinases.

The allele in repulsion with fire blight resistance, the homologue of the candidate fire blight resistance gene *MdE-EaK7*,

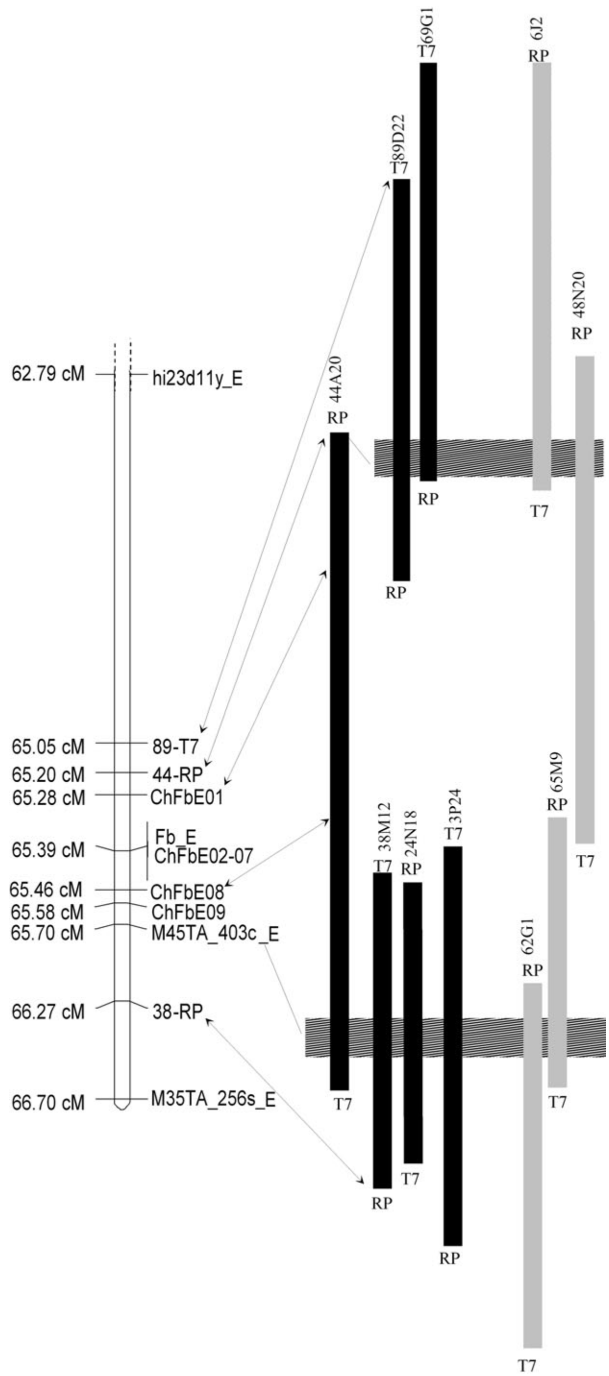

Fig. 1 Contig assembly with all bacterial artificial chromosome (BAC) clones originating from the resistant chromosome (black rectangles) and susceptible chromosome (grey rectangles) of apple cultivar 'Evereste', and genetic map of the end (bottom part) of the linkage group 12 of 'Evereste'. Genetic distances are in centimorgan (cM). The positions of the markers developed from BAC end sequences and of flanking microsatellite markers are shown with arrows. The dashed boxes indicate the region of the contig in which the M45TA or 44RP probes, used to screen the BAC library and to confirm the overlap of the clones, hybridized. T7, T7 end; RP, RP end of the BAC inserts; ChFbE02-07, microsatellite markers from ChFbE02 to ChFbE07.

MdE-EaK7susc (*MdE-EaK7* susceptible), was completely sequenced and investigated for the presence of the two characterizing signatures. *MdE-EaK7susc* encoded a protein consisting of 382 amino acids, which also possessed the Ser/Thr kinase signature and ATP-binding signature. At the nucleotide level, the identity between the two allelic versions was 98%. *MdE-EaK7susc* exhibited only 14 amino acids difference from *MdE-EaK7*, thus giving an amino acid identity of 96% (Fig. 4).

The unique predicted CC-NBS-LRR protein was analysed for the CC domain, Walker A, Walker B and kinase 3a motifs, LRR motif and other conserved motifs (van der Biezen and Jones, 1998; Hammond-Kosack and Jones, 1997; Traut, 1994). The *MdE-EaN* gene encoded for a 968-amino-acid protein possessing a putative CC domain (80% probability; Lupas *et al*., 1991), an NBS with Walker A, Walker B and kinase 3a motifs, but also an homeodomain (HD) motif and motifs 1–5. The LRR domain consists of 11 or 12 imperfect LRR motifs [LxxLxxLxxLxLxxxx(x) Lxx(x)L].

On the susceptible BAC clone, the *MdE-EaN* homologous susceptible gene, *MdE-EaNsusc* (*MdE-EaN* susceptible), was also found. The gene was also completely sequenced and investigated for the presence of the characteristic motifs. It encoded a 968-amino-acid protein and, like *MdE-EaN*, possessed a CC domain, Walker A, Walker B and kinase 3a motifs, HD motif and motifs 1–5; the LRR domain also possessed 11 or 12 imperfect motifs. At the nucleotide level, the identity between the two allelic versions was 98%, whereas, on the amino acid level, the allelic versions differed only in 26 single amino acid mutations, giving an amino acid identity of 97% (Fig. 5).

Verification of transcription of candidate resistance genes

All the primer pairs (Table 6) tested with the resistant 44A20 BAC clone amplified an amplicon of the expected size, whereas, with the susceptible 48N20 BAC clone for the *MdE-EaK2* primers, no amplification was obtained; *MdE-EaK3* primers amplified an additional 1200-bp amplicon, and the *MdE-EaK4* primers amplified a fragment of 1200 bp instead of 116 bp (Table 7). The sequencing of these amplicons made it possible to identify single nucleotide polymorphisms between the resistant and susceptible alleles (data not shown).

Reverse transcription-polymerase chain reaction (RT-PCR) amplicons were obtained for all candidate genes, but with a very weak intensity for *MdE-EaK1* and *MdE-EaK4* (for this reason, the amplicons of these two genes, visible in Fig. 6, were further amplified with the Qiagen, Hombrechtikon, Switzerland multiplex PCR Kit). The sequencing of all of these amplicons and the comparison with the sequences obtained from those of resistant and susceptible BAC clones confirmed the transcription of all alleles in coupling with resistance, whereas only the

Table 2 Microsatellite markers developed from the sequence of bacterial artificial chromosome (BAC) clone 44A20.

SSR name	Primer forward	Primer reverse	Size of amplicon in coupling with the resistance (bp)
ChFbE01*	5′-TTCAAGTCCCTGCATTTCAC-3′	5′-CAAGCTCATTGACCAGTTCG-3′	266
ChFbE02	5′-ACAGGGTGAAGAAAGGCATC-3′	5′-TTTCATCAAGCCTCTCATTGG-3′	248
ChFbE03	5′-CCGTTAATAAACTATTCAAACTCAGG-3′	5′-TGTACCGACATTTCCATACCG-3′	385
ChFbE04*	5′-TCCTTATCCCACACCAATTTC-3′	5′-AGAAGAACCATCTCTGTCTCCTC-3′	320
ChFbE05	5′-CATGTAAAGCCCATCTTCACC-3′	5′-CGACGAGGGCTTAGATTCTG-3′	338
ChFbE06*	5′-TTACCACCGTCCAAATCTCA-3′	5′-AATAATTGAAGGGTTGTGTGGA-3′	273
ChFbE07	5′-GAGCGCAATGACGTTCTAGG-3′	5′-TTGCAGAAGGCATTGTATCG-3′	272
ChFbE08	5′-AGATGGATCAAACCGGAGTC-3′	5′-TGAACTTCACGTGCTTGGTC-3′	267
ChFbE09	5′-TGATTACACACAAACCGAAGG-3′	5′-TTGAGGAAGAGAGGGAAGGAG-3′	272

SSR, simple sequence repeat.
Forward primers were extended at the 5′ end with the sequence 5′-GACTGCGTACCAATTCAAA-3′ after Schuelke (2000).
*Multilocus markers.

Table 3 Genotypes of the recombinant individuals showing a crossing over in the 165-kb region between the 44-RP and M45TA_403c_E markers.

	44RP	ChFbE01	ChFbE02	ChFbE03	ChFbE04	Fb_E	ChFbE05	ChFbE06	ChFbE07	ChFbE08	ChFbE09	M45TA_403c_E
	0 kb	39.1 kb	52.1 kb	78,8 kb	82.2 kb		87.7 kb	96.8 kb	109.4 kb	117.2 kb	137.5 kb	165 kb
ME3-049	R	S	S	S	S	S	S	S	S	S	S	S
ME3-721	R	S	S	S	S	S	S	S	S	S	S	S
EM1-489	R	S	S	S	S	S	S	S	S	S	S	S
EM1-501	R	S	S	S	S	S	S	S	S	S	S	S
ME3-901	R	R	S	S	S	S	S	S	S	S	S	S
ME-133	R	R	S	S	S	S	S	S	S	S	S	S
ME2-068	R	R	S	S	S	S	S	S	S	S	S	S
EM1-113	R	R	R	R	R	R	R	R	R	S	S	S
EM1-387	R	R	R	R	R	R	R	R	R	R	S	S
ME2-470	R	R	R	R	R	R	R	R	R	R	S	S
ME2-306	R	R	R	R	R	S/R	R	R	R	R	R	S
ME3-929	R	R	R	R	R	R	R	R	R	R	R	S
ME3-502	R	R	R	R	R	R	R	R	R	R	R	S
ME3-373	S	S	S	S	S	S	S	S	S	R	R	R
ME3-016	S	S	S	S	S	S	S	S	S	S	R	R

For each marker, the physical distance from the 44-RP end on the 44A20 bacterial artificial chromosome (BAC) clone is indicated in kilobases (kb) below the marker name. S, alleles in repulsion with Fb_E resistance (light grey boxes); R, alleles in coupling with Fb_E resistance (dark grey boxes). Fb_E, phenotype of the plants (R, resistant; S, susceptible). Fb_E could be positioned elsewhere within the interval ChFb01 and ChFb08 (first two markers not co-segregating with resistance).

transcription of *MdE-EaK1susc*,*MdE-EaK3susc*, *MdE-EaNsusc* and *MdE-EaK6susc* was observed (Table 7 and Fig. 6).

DISCUSSION

The clear phenotype of the major part of the population and the availability of closely linked markers (G. Parravicini *et al*., unpublished work), leading to the precise localization of QTL in a small interval (3 cm), was an excellent starting point for an attempt at positional cloning. In this work, a large BAC clone comprising the entire fire blight resistance locus was identified. The resistance region was further restricted by developing additional markers using the whole sequence of the BAC clone identified. Finally, the resistance QTL was assigned to a region of 78 kb delimited by ChFbE01 and ChFbE08 markers (corresponding to five recombinant individuals). The recombinant individuals were not scattered randomly throughout the resistant BAC clone. The average recombination frequency of the region located between markers M45TA_403c_E and 44-RP was one recombinant/11 kb. Assuming the above-mentioned recombination frequency for this region, a cold spot and hot spots of recombination were identified. Hot spots of recombination were found between the markers ChFbE08 and ChFbE09, where three instead of 1.8 recombinant individuals were found, between markers ChFbE07 and ChFbE08, with the presence of two instead of 0.7 recombinant individuals, and between markers ChFbE01 and ChFbE02, with three instead of 1.2 recombinant individuals. A cold spot of recombination was identified in the 57-kb region between ChFbE02 and ChFbE07, in which 5.2 recombinants were predicted but no recombinant individuals were present. For this

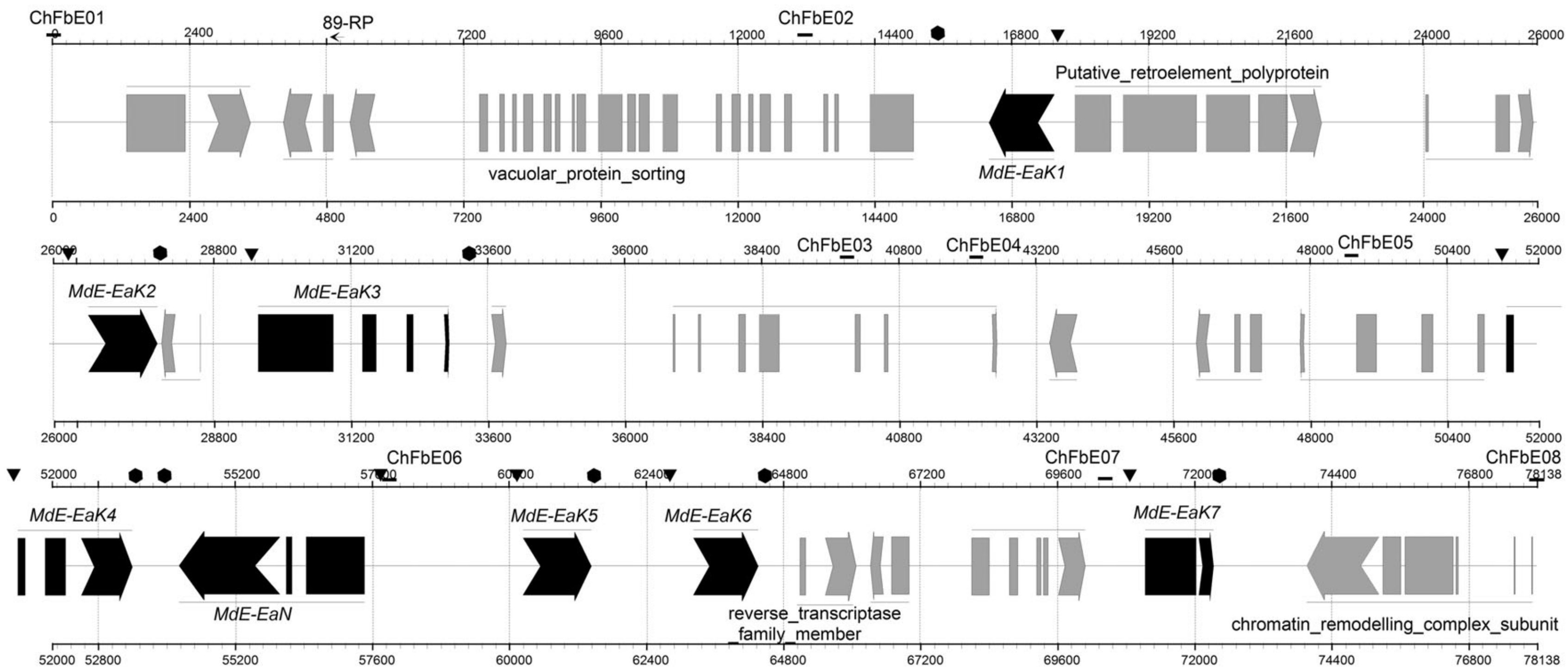

Fig. 2 Graphical output created using GFF2PS software (Abril and Guigo, 2000) and edited using DESIGNER 9.0 (Micrografx, Inc.) of all open reading frames (ORFs) predicted by FGENESH found on the 78-kb resistant region of bacterial artificial chromosome (BAC) 44A20 (Salamov and Solovyev, 2000). Exons are illustrated with boxes; single genes and the last exon of a group are shown by thick arrows. The genes showing similarities to resistance genes known to induce resistance to bacterial disease are highlighted in black. At the top of the sequence, boxes were added indicating the positions of mapped microsatellite markers; triangles indicate the transcriptional starts and hexagons indicate the transcriptional ends of the interesting ORFs.

Table 4 Amino acid percentage identity predicted by the CLUSTALW2 program (Larkin *et al*., 2007) between each of the protein kinases found on the restricted fire blight resistance region.

	MdE-EaK1	MdE-EaK2	MdE-EaK3	MdE-EaK4	MdE-EaK5	MdE-EaK6	MdE-EaK7	Length (amino acid)
MdE-EaK1	—							381
MdE-EaK2	80	—						402
MdE-EaK3	52	50	—					584
MdE-EaK4	77	74	44	—				462
MdE-EaK5	80	79	51	71	—			397
MdE-EaK6	79	76	53	71	79	—		378
MdE-EaK7	74	83	51	69	84	76	—	381

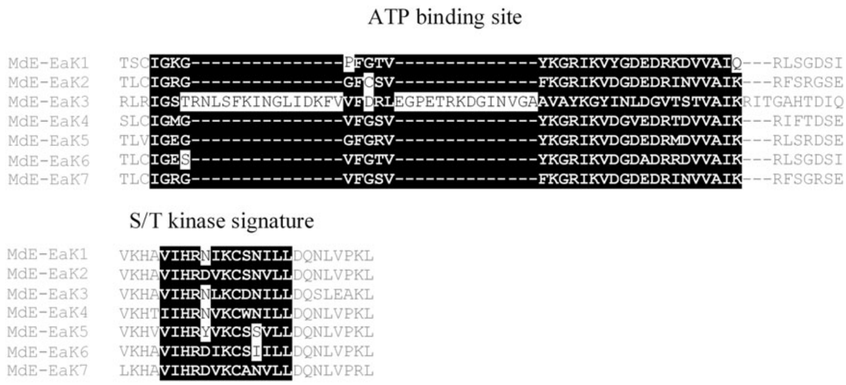

Fig. 3 Alignment of the amino acids encoded in the ATP-binding region and serine/threonine (Ser/Thr) kinase region of the seven putative kinases found by the CLUSTALW2 program (Larkin *et al*., 2007). Conserved amino acids of the Ser/Thr kinase and ATP-binding signatures are shown in black boxes.

Table 5 Summary of the main characteristics of the ATP-binding region signature and serine/threonine kinase region signature of the protein kinases found.

	MdE-EaK1	MdE-EaK2	MdE-EaK3	MdE-EaK4	MdE-EaK5	MdE-EaK6	MdE-EaK7
ATP-binding region signature	0	0	0	1	1	0	1
	P^5, Q^{29}	C^7	Various insertions	—	—	S^4	—
Serine/threonine kinase region signature	0	1	0	0	0	0	1
	N^5	—	N^5	N^5	Y^5, S^{10}	I^{10}	—

0, feature absent; 1, feature present. The amino acids not conserved are shown with the number indicating their position on the signature domain as obtained by expasy prosite (Hulo *et al*., 2008).

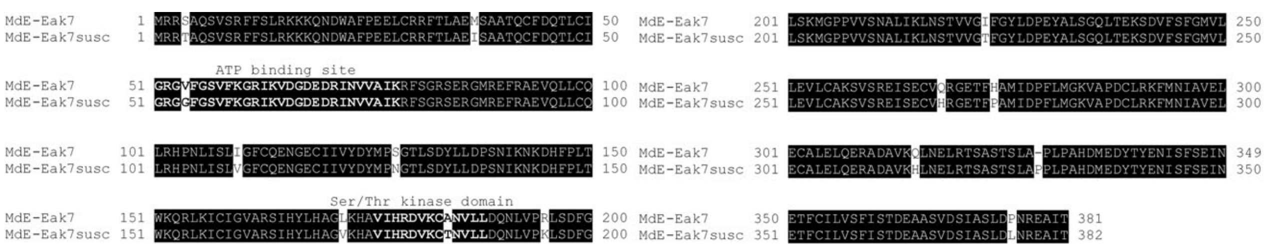

Fig. 4 Alignment of the amino acids encoded by *MdE-EaK7* and *MdE-EaK7susc* predicted by the CLUSTALW2 program (Larkin *et al*., 2007). Conserved amino acids are shown in black boxes; amino acids of the ATP-binding region and Ser/Thr kinase domain are shown in bold.

reason, it seems unlikely that the resistance region could be further narrowed without adding several thousand additional individuals to the population.

With a second hybridization, a BAC clone carrying the genomic region homologous to the resistant region on the susceptible chromosome was identified, which allowed a comparison of the resistant and susceptible alleles. The gene cluster of the 78-kb region appears to be unique in the apple cv. 'Evereste' genome, as a probe based on part of the *MdE-EaK7* gene only identified three additional BAC clones that could be assigned to the 78-kb region as either subclones of the BAC clone 44A20 or 48N20, with two BAC clones attributed by the SSR marker ChFbE06 to the susceptible chromosome and one BAC clone to the resistant chromosome. The *in silic*o analysis of the 78-kb

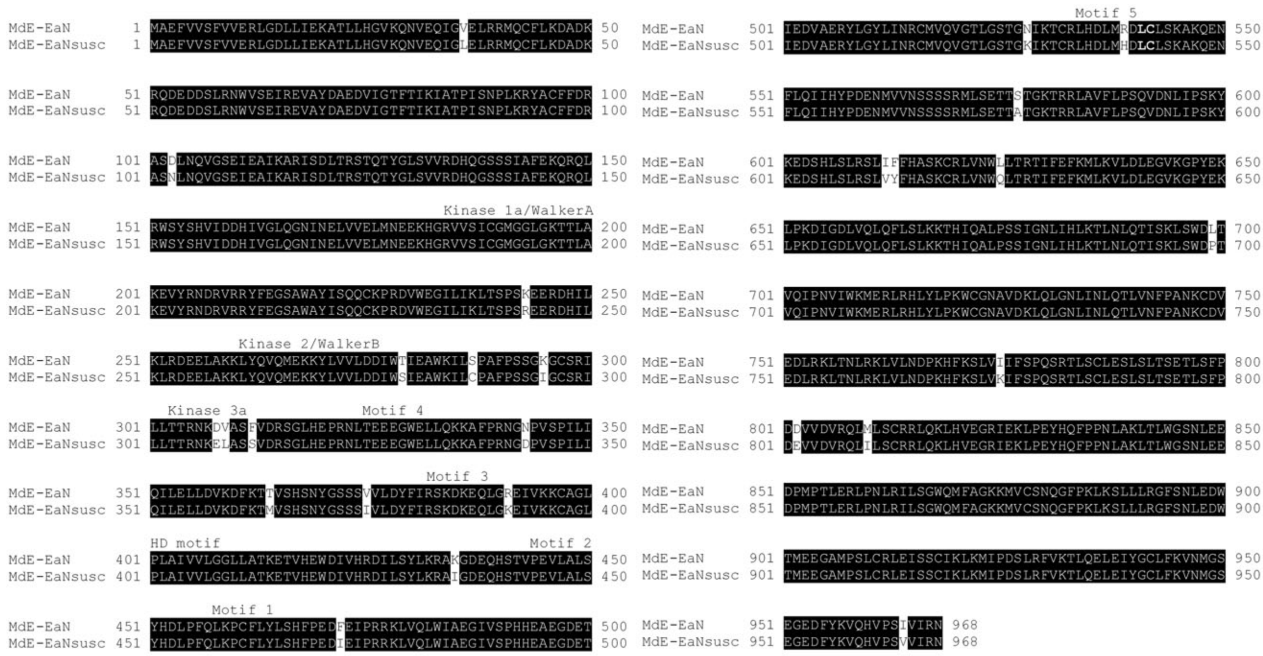

Fig. 5 Alignment of the amino acids encoded by *MdE-EaN* and *MdE-EaNsusc* predicted by the CLUSTALW2 program (Larkin *et al*., 2007). Conserved amino acids are shown in black boxes.

Table 6 Specific primers used to verify transcriptions of candidate resistance genes and length of the resulting amplicons.

Open reading frame	Forward primer	Reverse primer	Amplicon length (bp)
MdE-EaK1	5′-AGAGTATGCATCGTCTGACA-3′	5′-AGCTACTTTTCCCGCCAGA-3′	178
MdE-EaK2	5′-ACATGCCGGATCAGTAAATG-3′	5′-GCTCAATGAGTTGGGGAAAA-3′	153
MdE-EaK3	5′-AGCAGGGACGGGAGTTTAAGG-3′	5′-GGAAGTCGGAGAGGAAACCATTAG-3′	147
MdE-EaK4	5′-GGGTTGAGGACAGGACAGACG-3′	5′-AGCACAGCAATACCAAACTAAGCC-3′	116
MdE-EaN	5′-GCAGTTAGGGAGGGAAATTGTC-3′	5′-CCTTTGGCTCGCTTTAGATACG-3′	147
MdE-EaK5	5′-TGCCTCATCAGTGCTAAAAG-3′	5′-CTCAAGGAGTTGGGGACAAG-3′	153
MdE-EaK6	5′-ATAGAAAGCCCGGACGGCA-3′	5′-CAAGTTCCACCTGCACTTCA-3′	172
MdE-EaK7	5′-AATTAAACTCCACGGTGGTC-3′	5′-TGGAAAGTCTCACCCCTTTG-3′	166

Table 7 Transcription of gene alleles in coupling/repulsion with fire blight resistance.

Open reading frame	Amplicon size (bp)	Amplification on DNA of resistant BAC	Amplification on DNA of susceptible BAC	Amplification on cDNA of leaf extraction	Gene expression evidenced from the resistant allele	Gene expression evidenced from the susceptible allele
MdE-EaK1	178	+	+	+	+	+
MdE-EaK2	153	+	–	+	+	?
MdE-EaK3	147	+	+ and 1200 bp	+	+	+
MdE-EaK4	116	+	1200 bp	+	+	–
MdE-EaN	147	+	+	+	+	+
MdE-EaK5	153	+	+	+	+	–
MdE-EaK6	172	+	+	+	+	+
MdE-EaK7	166	+	+	+	+	–

BAC, bacterial artificial chromosome; +, amplification or transcription of ORF; –, ORF not amplified or transcribed; ?, not possible to sequence owing to the absence of amplification.

sequence revealed the presence of 23 ORFs, eight of which appeared to be more interesting because of their homology to known resistance genes (seven kinases and one CC-NBS-LRR). For seven, the alleles in repulsion with the resistance could also be identified. It was not possible to assess the presence of the susceptible allele of the *MdE-EaK2* gene as no amplicon could be produced with the specific primers. This could be a result of the absence of the susceptible allele or to the mutation of the primer

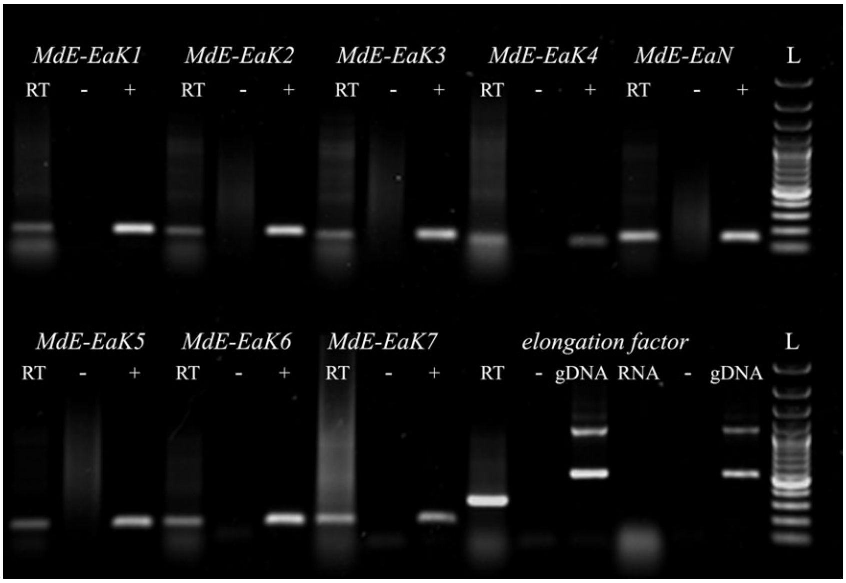

Fig. 6 Amplification patterns of *MdE-EaKs* and *MdE-EaN* genes. +, bacterial artificial chromosome (BAC) 44A20 DNA used as template; –, negative control (double-distilled H_2O); RT, cDNA used as template; gDNA, 'Evereste' genomic DNA used as template; L, 100-bp DNA ladder (O'GeneRuler™ 100 bp Plus DNA Ladder, Fermentas).

sites in the susceptibility region. All eight ORFs of the resistant region were transcribed in the resistant cv. 'Evereste', characterizing them as genes. From the susceptibility region, however, only the homologous susceptible ORFs *MdE-EaK1susc*, *MdE-EaK3susc*, *MdE-EaNsusc* and *MdE-EaK6susc* were transcribed (Fig. 6).

Only MdE-EaK7 strictly possessed both Ser/Thr kinase and ATP-binding signatures (Fig. 3 and Table 5) and, more interesting, only the resistant allelic version of the gene was expressed (Table 7), leading us to believe that this gene is the first candidate fire blight resistance gene. Nevertheless, a second candidate, MdE-EaK2, was also very close to exhibiting both signatures as, in the ATP-binding region, a unique amino acid mutation disrupted the predicted conserved pattern. In fact, the expected glycine (G^7) was replaced by another small and hydrophobic amino acid, cysteine (C^7). In addition to MdE-EaK7 (and MdE-EaK2), another interesting gene was transcribed in both its allelic versions (Table 7), namely MdE-EaN. MdE-EaN is predicted to encode a region of LRRs, a putative NBS and a CC sequence. NBS-LRRs are a family of proteins also comprising Prf of tomato and RPM1 and RPS2 of *Arabidopsis* (Bent *et al*., 1994; Grant *et al*., 1995; Salmeron *et al*., 1996)

Both proteins, MdE-EaN and MdE-EaK7, show a high homology with two other proteins involved in bacterial resistance, Prf and Pto from tomato, respectively (Martin *et al*., 1993; Mucyn *et al*., 2006; Salmeron *et al*., 1996). These two proteins are in fact involved in the resistance to races of *P. syringae* pv. *tomato*, a bacterium causing bacterial speck on tomato. The genomic organization of the resistance region in both apple and tomato species reveals intriguing similarity: like *MdE-EaN* and *MdE-EaK7*, *Prf* and *Pto* also comprise a cluster of genes, formed in this case by six protein kinase encoding genes and an NBS-LRR gene on chromosome 5 of tomato (Chang *et al*., 2002; Martin *et al*., 1991). This is not the first report of an analogy between apple and tomato. During the map-based cloning of *Vf*, the gene conferring resistance to apple scab, Vinatzer *et al*. (2001) identified a cluster of three genes homologous to the *Cladosporium fulvum* resistance gene family of tomato, named *HcrVf*, and Broggini *et al*. (2009) identified at least another six genes homologous to the *C. fulvum* resistance genes on LG1 and LG6 of the apple cv. 'Florina'. In addition, in tomato, these genes were found only on two chromosomes, namely chromosomes 1 and 6.

Both MdE-EaK7 and Pto present all the characteristic domains conserved in a Ser/Thr kinase protein. In addition, MdE-EaN possesses the same functional domain identified on Prf, with the exception of the long N-terminal domain, which, in Prf, was found to interact with Pto (between amino acids 1 and 546; Mucyn *et al*., 2006). However, the CC domain of MdE-EaN may possess the same functionality as the RIN4-interacting N-terminal CC domain of the *Arabidopsis* RPM1 protein (Mackey *et al*., 2002). For this reason, an interaction between MdE-EaN and MdE-EaK7 remains possible, but must be confirmed *in vivo*. To indicate which gene is most important, it could also be interesting to perform an association study, but, in this case, this is not very feasible. Indeed, more than 2703 individuals have already been used in this study and, in addition, the strong homology between the homologue kinase genes creates great difficulty in discriminating these genes between different cultivars. At the nucleotide level, these genes possess a homology up to 91% that renders it very difficult to discriminate the homologues between the two alleles in the resistant parent 'Evereste'. A trial of gene identification on the susceptible parent MM106 was made using the same primer pairs as employed to detect transcription in the resistant parent 'Evereste' (the cultivar in which they were designed). Several amplicons were found after amplification on MM106 for each primer pair. Therefore, after sequencing, it was not possible to identify which amplicon was allelic to those used for primer design.

Therefore, the inability of primer pairs based on the resistant 'Evereste' allele to identify the alleles present in other cultivars, combined with the similarities within the kinase family, complicate efforts to perform association genetics.

The way in which the Pto–Prf complex acts has been well studied: Pto monitors the interaction between the *AvrPto* or *AvrPtoB* effector of *P. syringae* and Prf; in the case of an interaction between Pto and AvrPto or AvrPtoB, Pto will autophosphorylate itself and activate Prf, and a further Ser/Thr kinase, Pti1, which induces a mitogen-activated protein kinase (MAPK) cascade, resulting in a hypersensitive response (Wu *et al*., 2004; Zhou *et al*., 1995, 1997). It has been suggested by Mucyn *et al*. (2006, 2009) that Pto and Prf act as a molecular switch, controlling immune signalling, in which Pto keeps Prf in an inactive state until the recognition of the *P. syringae* effectors, and that these effectors have evolved to inhibit the Pto–Prf complex by targeting the co-regulatory interface. We can therefore hypothesize a similar mode of action in the pathosystem *E. amylovora*–*Malus*. In this case, MdE-EaN, like Prf, could act as the 'guard' protein that triggers resistance, whereas MdE-EaK7, like Pto, could act as the 'guardee' following the guard hypothesis (Dangl and Jones, 2001), interacting with a bacterial Avr effector which remains to be identified.

Therefore, to ultimately validate fire blight resistance functionality, the candidate resistance genes must first be subjected to complementation analysis on susceptible plants or silencing analysis on resistant plants.

EXPERIMENTAL PROCEDURES

Plant material, DNA extraction and selective pathological tests

The populations ME and ME2 were the same as in G. Parravicini *et al*. (unpublished work); 1192 individuals from a third MM106 × Evereste cross (ME3) and 817 individuals from a reciprocal Evereste × MM106 cross (EM1) were added in year 2008 to reach a total of 2703 progeny individuals. DNA was extracted from lyophilized leaves following a cetyltrimethylammonium bromide (CTAB) extraction procedure (Aldrich and Cullis, 1993). All individuals were genotyped for markers CH03c02_E, Hi07f01_E, Hi23d11y_E, M45TA_403c_E and M35TA_256s_E, the last two markers having been developed to encompass the QTL region (G. Parravicini *et al*., unpublished work). All recombinant individuals between markers M35TA_256s_E and Hi23d11y_E were assessed for fire blight resistance as described in Durel *et al*. (2009), with up to 20 grafted copies per individual for the most strategic ones (i.e. individuals recombining close to the QTL peak). The quantitative phenotypic data were transformed into binary data (i.e. resistant : susceptible) to be used for single gene mapping of the Fb_E locus. As phenotyping was repeated in 2008, 2009 and 2010, a nonambiguous binary classification was finally possible for all strategic recombinant individuals, except one.

Genomic library construction of cultivar 'Evereste' and chromosome landing strategy

An apple cv. 'Evereste' BAC library was prepared with high-molecular-weight genomic DNA processed at Amplicon Express Inc. (Pullman, WA, USA), as in Tao *et al*. (2002). The partially digested *Hin*dIII DNA was then cloned into pCC1BAC® vector (EPICENTRE, Madison, WI, USA), DH10B *Escherichia coli* cells were transformed, arrayed on 384-well plates and spotted on Hybond N+ membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

The chromosome landing strategy consisted of a unique hybridization, with a probe linked to the resistance locus that identifies BAC clones covering the entire resistance region, flanked by two non-co-segregating markers. The positive BAC clones were subjected to fingerprints and PCR to confirm that they belonged to the contig under construction, which was oriented using markers developed on the BAC extremities. A probe, named M45TA, derived from the nearest marker to the resistance QTL peak, M45TA_403c_E (G. Parravicini *et al*., unpublished work), was used to screen the *Hin*dIII BAC library, as explained in Galli *et al*. (2010), with the exception of the BAC ends, which were sequenced using RP or T7 primers (Synergene Biotech GmbH, Zurich, Switzerland). Markers developed from the BAC ends were then tested for polymorphisms. A specific PCR was performed in a volume of 20 μL containing 5 ng DNA (parental and recombinant individuals), 0.07 U of Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA), 1 × provided buffer, 0.1 mM deoxynucleoside triphosphates (dNTPs) (Fermentas, St. Leon-Rot, Germany) and 0.2 μM each of newly designed forward and reverse primers. Amplifications were executed in a TProfessional Basic Thermocycler (Biometra, Goettingen, Germany) with the following cycle: 2 min at 94 °C, then 10 cycles of 30 s at 94 °C, 30 s at 65 °C and 1 min at 72 °C, followed by 20 cycles of 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C, with a final elongation step of 10 min at 72 °C. PCR products were run on 1% agarose gel and visualized by staining with ethidium bromide.

To identify the BAC clone with the region in repulsion with fire blight resistance, a probe derived from one of the protruding BAC ends was used.

BAC sequencing

The BAC clone encompassing the entire resistance region was sequenced at the Genome Center at Washington University, St.

Louis, MO, USA. The sequences obtained were analysed with the PC software SEQUENCER (Genecodes Corporation, Ann Arbor, MI, USA); a contig was created with the following parameters: Clean Data, a minimum overlap of 20 bases and a minimum match percentage of 99.

Fine mapping of fire blight resistance region

Microsatellite sequences were identified using the online SPUTNIK software (SPUTNIK: DNA microsatellite repeat search utility by Chris Abajian at Washington University) and, considering their positions, pairs of primers flanking microsatellite repeats were designed. Microsatellite markers were named with the recognition letters of the country in which the work was performed (Ch, Switzerland), the recognition letters of the plant disease considered (Fb, Fire blight), the initial letter of the apple cultivar in which the microsatellite was found (E, 'Evereste') and a number. A specific PCR was performed following Schuelke (2000) in a volume of 22 μL with 50 ng DNA (parental and recombinant individuals), 0.07 U of Taq DNA Polymerase, 1 × provided buffer, 0.1 mM dNTPs, 0.16 μM forward-labelled E31 (5′-GACTGCGTACCAATTCAAA-3′), 0.04 μM forward and 0.16 μM reverse primers. The following procedure was applied: 5 min at 94 °C, then 30 cycles of 30 s at 94 °C, 45 s at 56 °C and 45 s at 72 °C, followed by eight cycles of 30 s at 94 °C, 45 s at 53 °C and 45 s at 72 °C, with a final elongation step of 10 min at 72 °C. All the amplifications were performed in a TProfessional Basic Thermocycler (Biometra) and then run on an ABI 3130xl DNA sequencing system (PE Applied Biosystems, Foster City, CA, USA) using the fragment analysis protocol provided with the following modification: 50-cm array and POP7 polymer. The markers were analysed using GENEMAPPER4 (PE Applied Biosystems) with Gene Scan 500 LIZ as size standard (PE Applied Biosystems). Fine mapping was performed using JoinMap version 3 (Van Ooijen and Voorrips, 2001).

Identification of candidate fire blight resistance genes

The open reading frames (ORFs) and transcriptional starts and ends present in the narrowed resistance region were predicted with FGENESH using the tomato genome as a reference (Salamov and Solovyev, 2000), a graphical output was created using GFF2PS software (Abril and Guigo, 2000) and edited with DESIGNER 9.0 software (Micrografx, Inc., Dallas, TX, USA). Analysis of the predicted proteins and identification of the putative conserved domains and functional sites were performed with BLASTP [available on-line from the National Center for Biotechnology Information (NCBI) server], expasy prosite (Hulo *et al*., 2008) and COILS servers (Lupas *et al*., 1991). Alignments were made using the CLUSTALW2 program (Larkin *et al*., 2007).

Verification of transcription of candidate resistance genes

Specific primers were designed, aligning the candidate resistance gene sequences of the cluster to the identified unique regions. These combinations of primers were used first to amplify the alleles present on the BAC clones corresponding to the resistance and susceptibility regions. The specific PCRs were performed in a volume of 20 μL containing 2 μL BAC DNA, 0.07 U of DreamTaq DNA Polymerase (Fermentas), 1 × provided buffer, 0.1 mM dNTPs and 0.2 μM of specific primers. Amplifications were performed in a Perkin-Elmer Cetus Gene Amp PCR System 9600 (Perkin-Elmer, Waltham, MA, USA) with the following cycle: 2 min at 94 °C, then 35 cycles of 30 s at 94 °C, 1 min at 60 °C and 1 min at 72 °C. PCRs were run on 1% agarose gels containing traces of ethidium bromide, and sequenced to identify polymorphisms.

RNA was extracted from approximately 40 mg of leaf tissue of uninfected apple cv. 'Evereste' with Concert™ Plant RNA Reagent (Invitrogen, Gaithersburg, MD, USA), following the manufacturer's instructions, but with the following modifications: isopropyl alcohol was cooled to –20 °C prior to use, and the incubation time after its addition was made at –20 °C instead of at room temperature. The RevertAid™ H^- First Strand cDNA Synthesis kit (Fermentas) was used to create cDNA, following the manufacturer's instructions, employing Oligo $d(T)_{18}$ for first strand synthesis. Expression of the susceptible and resistant alleles was verified using the same primers as before on cDNA and sequencing the amplicons obtained. Primers spanning an intron on apple elongation factor *EF1* α gene were used to check the presence of cDNA and possible genomic DNA contamination on RNA extraction, as described in Szankowski *et al*. (2009).

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